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Advanced techniques for characterizing bioinspired materials

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Advanced techniques for characterizing bioinspired materials

Abstract

Understanding materials' analytical techniques is a key feature to ensure efficient material development and to fully evaluate their functional properties. Biobased materials are emergent materials that are attracting attention due to their outstanding properties, pushing forward their use in a wide range of applications going from biomedical and pharmacy to even food industry, among others. This chapter provides an educational resource with the most relevant characterization techniques that are applicable to the development and characterization of biobased materials. It discusses physical, thermal, and chemical characterization techniques that are able to provide information related to the microstructure and morphology, and the mechanical, thermal, and chemical properties of materials. Additionally, it provides cues and considerations for the characterization techniques, complemented with relevant case studies and examples from different biobased materials.

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Advanced techniques for characterizing bioinspired materials

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Abstract

Understanding materials analytical techniques is a key feature to ensure efficient material development and to fully evaluate their functional properties. Biobased materials are emergent materials that are attracting attention due to their outstanding properties, pushing forward their use in a wide range of applications going from biomedical and pharmacy to even food industry, among others. This chapter provides an educational resource with the most relevant characterization techniques that are applicable to the development and characterization of biobased materials. It discusses physical, thermal and chemical characterization techniques that are able to provide information related to the microstructure and morphology, and the mechanical, thermal and chemical properties of materials. Additionally, it provides cues and considerations on the characterization techniques, complemented with relevant case studies and examples from different biobased materials.

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Introduction

Materials science can be defined as the study of the relationship between materials processing, its structure and utterly, its properties will be affected by the interplay of these parameters (figure 1). Materials characterization is a powerful tool to understand how the different parameters involved in the early stages of design and processing, influence their structure and performance.

The next sections provide a brief discussion of the most useful characterization techniques to understand material basic properties; from its mechanical performance and fatigue-cycle studies to its chemical, morphological structure and thermal properties.

At the end of the chapter, we provide some suggestions of further readings for a better comprehension of the materials properties, as well as to assist the design of novel processing methodologies and characterization techniques that can be used to explain and correlate materials features with their potential applications.

Figure 1 – Relationship between the materials properties and the characterization.

Mechanical properties

The mechanical properties are one of the most important physical properties to assess in order to understand a material performance, not only at laboratorial scale but, more importantly, to explain and predict its behaviour in daily applications.

In order to discuss the quantitatively mechanical performance, it is necessary to derive mathematical expressions that relate the stress (σ) and strain (ε). When a material with uniform cross-section is stretched under a unidirectional stress, the overall sample volume will remain constant. Nevertheless, some changes occur in different directions. As it can be observed from figure 2, when the material is stretched under a tensile stress, the length of the material increases, while the dimensions in the other directions (Y and Z) decrease. The change in sample length in the stress (σ) direction is called deformation (ε) and is given by:

$$\varepsilon = \frac{\Delta L}{L_0} = \frac{L - L_0}{L_0} \quad (1)$$

where $\Delta L = L - L_0$, being L the length after deformation, and L_0 the initial length of the sample.

Considering that an ideal elastic solid obeys Hooke's law:

$$\sigma = E\varepsilon \quad (2)$$

where E is known as the material *Young's modulus* or *elasticity modulus*, the material stress can be obtained by:

$$\sigma = \frac{F}{A} \quad (3)$$

where F is the force applied to the material and A is the cross-sectional area of the sample, given by $A = t * w$, being t the thickness and the w the width of the sample.

When a material is stretched, one of the dimension's increases and the other two will decrease. The ratio between the changes in the linear strain produced in the perpendicular direction (ε_{perp}), due to the tensile stress producing the tensile strain (ε), is called the Poisson's ration (ν), and is defined by:

$$\nu = -\frac{\varepsilon_{perp}}{\varepsilon} \quad (4)$$

The minus signal is introduced in order to make ν positive for most of the materials, for which ε_{perp} has the opposite sign to that of the tensile strain (Bower, 2002).

According to the Hooke's law (equation 2), the application of a stress leads to an instantaneous response strain of the material and once the stress is removed, the strain instantaneously reverts to zero. Nevertheless, polymeric materials may often deviate from this ideal behaviour, mainly because their mechanical response presents a (Bower, 2002, Sperling, 2006):

- i. *Time-dependence* of response;
- ii. *Non-recovery* of strain on removal of the stress, i.e. *yield*;

- iii. *Non-linearity* of response (ϵ is not proportional to σ), which does not imply no recovery;
- iv. *Large strains* without fracture;
- v. *Anisotropy* of response.

Figure 2 depicts the representative stress-strain (σ vs ϵ) plot for a polymeric material with the five main regions that can generally be identified during the mechanical measurement (Bower, 2002, Sperling, 2006):

- (1) When a constant stress is applied to a polymeric material, deformation starts to occur. The polymer chains that originally are randomly organized start to move in the direction of the applied stress, until reaching a maximum stress - the yielding point. While for small loads the material behaviour is linear viscoelastic, with increasing load the behaviour becomes progressively non-linear.
- (2) At the yielding point, the deformation becomes irreversible since the stress induces a plastic flow. The polymer chains start to disentangle and align in the direction of the applied mechanical stress with a necking region starting to appear in the centre of the sample.
- (3) An increase in the strain leads to a decrease of the stress due to polymer chain disentanglement and alignment in the direction of the applied strain. At this point, a visible necking region is possible to observe in the centre of the sample.
- (4) After the necking formation, the stress reaches a plateau, and in this region, the necking starts to grow towards the edges of the sample due to the continuous alignment of the polymer chains in the direction of the stress – this stage is called cold draw process, leading to highly-aligned polymer chains.
- (5) Due to the high alignment of the polymer chains, and in order to maintain the experimental strain rate constant, there is an increase of the stress until mechanical failure of the polymer; this stage is also known as strain-hardening.

Figure 2 – Representative stress-strain plot for a polymeric material. The numbers in parentheses represent the different stages that occur during mechanical experiment in polymeric samples.

The mechanical properties of some natural and synthetic polymers are presented in table 1.

Table 1 - Mechanical properties of some natural and synthetic polymer materials.

<i>Material</i>	<i>E</i> (MPa) ^(a)	<i>UTS</i> (MPa) ^(b)	<i>ε_{break}</i> (%) ^(c)	<i>Reference</i>
<i>Gelatin films</i>	20 ± 12	3 ± 1.5	25 ± 12	(Bigi et al., 2001)
<i>Silk-Elastin-Like Protein (film)</i>	3197 ± 326	70.5 ± 7.9	42.4 ± 22.3	(Machado et al., 2015)
<i>Silk-Elastin-Like Protein (random fibres)</i>	142 ± 48	14 ± 2	22 ± 6	(Raul et al., 2013)
<i>α-Elastin (random fibres)</i>	0.33 ± 0.01	*	*	(Araujo et al., 2014)
<i>Polyhydroxybutyrate</i>	3500	40	6	(Goodfellow, 2015)
<i>Poly(lactic acid)</i>	1610 ± 80	61.5 ± 3.80	5.2 ± 0.5	(Zhang and Sun, 2004)

^(a) *E* = elasticity modulus; ^(b) UTS = ultimate tensile strength; *ε_{break}* = strain-to-failure.

Figure 3 presents the general mechanical behaviour of different polymer materials. A polymer single-crystal fibre presents a very high tensile strength and a low strain at break, whereas an elastomer presents a very low tensile strength and high strain at break.

Figure 3 – Schematic stress-strain curves of different polymers, drawn approximately to scale (Bower, 2002).

Mechanical fatigue

Fatigue is one of the most important issues to address in materials science. Nevertheless, in the characterization of soft materials, it is a topic often underestimated and not completely explored. As the use of soft materials is growing fast, there is an emerging interest to understand, model, and predict the fatigue behaviour and life cycle of a material. Fatigue failure can be interpreted as the failure of a certain structure under the action of repeated fluctuating stresses or strains. These induce progressive, localized and permanent microstructural changes caused by cyclic plastic deformations that may

culminate in the formation of cracks and subsequent final fracture (Erber et al., 1993). Mathematical models were firstly developed to establish relationships with fatigue or damage accumulation, property degradation, loading variables and applied cycles (Bower, 2002). These revealed to be fairly reliable and simple to use in many circumstances, making them attractive to the design engineer for life cycle predictions of developed structures.

While the application of one small plastic strain does not cause remarkable changes in the microstructure of a material, multiple repetitions of the plastic strain can lead to cumulative damage which eventually results in fatigue failure (Anand and Parks, 2004). Therefore, fatigue experiments are performed by submitting the material specimens to a cyclic varying load or displacement (figure 4). The material can be submitted to different shapes of time variation for the applied stress or strain such a sine, triangular or other more complex wave geometries, depending on the application characteristics.

Figure 4 - Fatigue testing (Ashby and Jones, 2012).

In polymeric materials, the linear relationship between the stress and strain (elasticity) is only valid for very small deformations. Nevertheless, inelasticity is always present even though it may be detectable only by fine measurements (Gheorghe Frunz and Diaconescu, 2006). As a consequence of inelasticity, when a cyclic stress is applied, the plastic contribution for the mechanical response increases giving origin to a hysteresis closed loop during the cyclic mechanical loading (figure 5). This loop evolves with the number of stress cycles, its shape changing with increasing number of fatigue cycles (N), until finally fail (when $N = N_f$ – the number of cycles to failure) (Ashby and Jones, 2012). As the area within the loop equals the amount of energy dissipated in the material and since sustained hysteresis relates to the rate of damage accumulation, the study of the hysteresis loop offers useful information on a material state (Gheorghe Frunz and Diaconescu, 2006, Erber et al., 1993).

Figure 5 – Schematic of a mechanical hysteresis loop.

Coffin-Manson Model – plastic strain range – life model

The Coffin-Manson mathematical relationship has been used to model crack growth in metals due to repeated temperature cycling. This model establishes a relation between the width of hysteresis loops and the number of loading cycles required to produce failure; linking the hysteresis energy dissipation, the accumulation of material damage, and the average number of loading cycles leading to failure (Erber et al., 1993).

In order to determine the plastic strain range ($\Delta\varepsilon_p$), graphically represented by the width of the hysteresis loop (figure 5), the elastic strain range ($\Delta\varepsilon_e$) is subtracted from the total strain range ($\Delta\varepsilon_t$) (Kanchanomai and Mutoh, 2004). The relationship between the plastic strain range and the number of cycles to produce failure follows the following equation:

$$(N_f)^\alpha * \Delta\varepsilon_t = \theta \quad (5)$$

where N_f is the fatigue life, α is the fatigue ductility exponent, and θ is the fatigue ductility coefficient (Kanchanomai and Mutoh, 2004). The result of the low-cycle fatigue according to the Coffin-Manson model is near a straight line for most common materials.

A modified equation was proposed later, in order to model and predict the fatigue life for different experimental frequencies:

$$(N_f * v^{k-1})^\alpha * \Delta\varepsilon_t = \theta \quad (6)$$

where v and k are the frequency and frequency exponent, respectively, evaluated from the fatigue life-frequency relationship (Kanchanomai and Mutoh, 2004). According to the Coffin-Manson modified model, the relationship between the plastic strain range and frequency can be fit to a single curve. Nevertheless, the effect of the temperature in the material fatigue life is not predicted by this model, which implies the measurement of the fatigue parameter should be performed at desired temperatures.

Smith – Watson – Topper Model

According to the Smith-Watson-Topper (SWT) model, the fatigue life cycle for any situation of mean stress depends on the product between the maximum stress and the total deformation (Ashby and Jones, 2012):

$$\frac{\Delta \varepsilon_t}{2} = \varepsilon_a = \frac{\Delta \varepsilon_e}{2} + \frac{\Delta \varepsilon_p}{2} = \frac{\sigma_f'}{E} (2N_f)^b + \varepsilon_f' (2N_f)^c \quad (7)$$

where $\frac{\Delta \varepsilon}{2}$ is the strain amplitude, ε_f' is the fatigue ductility coefficient, c is the fatigue ductility exponent and E is the material Young modulus. In this model, the material fatigue is based on the strain-life approach, where the cyclic response of the material is within the elastic-plastic stress-strain range, and can only be used to predict the fatigue life at zero mean stress (Ince and Glinka, 2011).

Morrow energy model – plastic strain energy density-life model

The plastic strain energy density can be physically interpreted as the energy of distortion associated with the change in shape of a volume element and is related to failure, particularly under conditions of ductile behaviour, as it often occurs in many of the polymeric materials submitted to cyclic mechanical solicitation (Kanchanomai and Mutoh, 2004). According to the mathematical model proposed by Morrow (Materials, 1965), the strain energy density can be evaluated numerically as the inner area of the saturated hysteresis loop for the uniaxial fatigue experiments (figure 6), and is expressed mathematically as:

$$N_f^m * W_p = C \quad (8)$$

where W_p is the plastic density energy, N_f is the fatigue life, and m and C are the fatigue exponent and coefficient, respectively. Morrow's model was modified in order to predict the effect of experimental frequency in material fatigue life cycle, according to:

$$(N_f * v^{k-1})^m * W_p = C \quad (9)$$

where v and k are the frequency and frequency exponent, respectively, also proposed by the Coffin-Manson model for fatigue life-frequency relationship (Kanchanomai and Mutoh, 2004).

The above theories were developed to explain and predict mainly metallic materials fatigue life cycle, but they also proved to be suitable to predict polymers fatigue behaviour.

Scaffolds and membranes used for tissue and biomedical engineering are often under cyclic loading during *in vitro* or *in vivo* experiments. For example, poly- ϵ -caprolactone (PCL) macroporous scaffolds were produced by dissolving the polymer in a suitable solvent in the presence of a poly(methyl methacrylate) spheres as a sacrificial porogen (Panadero et al., 2013). The scaffolds were cut in discs with 6 mm diameter and thickness of approximately 2 mm, and submitted to a cyclic mechanical compression loading at a fixed strain of 15 %, in order to mimic the cartilage deformation during walking. Panadero et al. (2013) found that the number of mechanical load-unload cycles can increase up to 5 times in water-immersed samples with all pores filled with water, when compared with those in dry conditions. This increased performance was attributed to the more uniform stress distribution that occurs when the sample is filled with water, and also to the incompressibility of water that supports a significant part of the mechanical cyclic loading. In their work, fibrin and water were also used to fill the pores leading to similar results. Figure 6 represents the trabeculae PCL sample morphology with fibrin filling the pore structure, as well the mechanical loading-unloading cycles applied to the samples. Based on experimental data, and using the fitting parameters obtained from Morrow's model, the mechanical life cycle performance was calculated and compared to the experimental results. In this model, a perfect correlation occurs when the data points lie on the solid diagonal line, and when the experimental data deviates no more than 10 % from the theoretical line (Materials, 1965, Kanchanomai and Mutoh, 2004, Panadero et al., 2013). Their findings suggest that the Morrow's theoretical model can be used to explain the polymer fatigue life behaviour for the PCL trabecular scaffold under different conditions (figure 6).

Figure 6 – a) PCL sample microstructure with fibrin filling the pore structure, b) sample characteristic hysteresis loops with inset showing the cyclic mechanical loading applied, c) Relationship between the overall equivalent behaviour similar to plastic strain energy density and number of load-recovery cycles of PCL samples and d)

Comparison of predicted with experimentally fatigue behaviour calculated according to the Morrow's model (figure adapted from (Panadero et al., 2013)).

In another experiment, the PCL porous matrix was filled with poly(vinyl alcohol) (PVA) and showed that the presence of the cross-linked PVA inside of the PCL macropores leads to an increase of the Young's modulus (Panadero et al., 2015b). However, the mechanical fatigue behaviour predicted by the Morrow's model revealed that the PCL-PVA scaffolds had a poor performance when compared to the PCL samples filled with fibrin and water, likely due to complex changes in the porous structure and local interactions among the different phases, e.g. PCL, PVA and water.

Based on the previous results obtained for the different trabecular PCL scaffolds, the authors studied the effect of the mechanical fatigue cycle using murine bone marrow cell (KUM5 cells) culture. They applied a fixed strain of 15% at a frequency of 1 Hz during 30 min and 90 min of stillness and real-time PCR was performed to identify the cell expression of characteristic markers of several components of the extra cellular matrix (ECM) (Panadero et al., 2015a). The fatigue cycle measurements performed in the bioreactor showed that the mechanical behaviour of the PCL constructs with ECM produced by the cells is different from the one reported above (Panadero et al., 2015a) but the overall fatigue cycle can be described by the Morrow's model, which is an important finding that could help in future scaffold design.

Fourier transform infrared spectroscopy

Infrared radiation is an electromagnetic radiation with wavelengths (ν) between 0.7 – 500 μm , corresponding to radiations with wavenumbers ($\nu = 1/\lambda$) between 1.4×10^4 and 20 cm^{-1} , which specifies the number of waves per centimetre.

When a molecule is submitted to infrared radiation, the absorption of such radiation leads to vibrational and/or rotational movements. Infrared absorption occurs when the *ac* electric field frequency associated to the incident radiation matches the changes of frequency in the vibration or rotation movements of the molecule that is absorbing the electromagnetic radiation (Stuart, 2005). When this matching occurs, the electromagnetic radiation is absorbed, promoting a change in the molecule vibration amplitude, which also affects the dipolar moment during absorption - also called the *transition dipole* or *transition moment* (Bower and Maddams., 1989). The absorbed

energy is subsequently lost by the molecule, either in the form of re-radiation or, more usually, by being transferred to other molecules of the material in the form of heat energy (Bower, 2002).

Essentially, an infrared spectrometer consists of the following elements (Bower and Maddams., 1989):

- i) a source of radiation with a continuous spectrum over a wide range of infrared wavelengths;
- ii) a means of dispersing the radiation into its constituent wavelengths;
- iii) a sample holder, allowing the radiation to pass through the sample or to be reflected;
- iv) an infrared detector capable of measuring over the range of wavelengths of interest;
- v) an acquisition and recording system.

The most common system used to study polymers is the *Fourier-transform* spectrometer, where all wavelengths pass through the sample to the detector simultaneously. The total transmitted intensity is measured as a function of the displacement of one of the mirrors in a double-beam interferometer, usually of the Michelson type. The separation of the wavelengths is subsequently done mathematically, by employing a Fourier transform on the intensity versus displacement data (Bower and Maddams., 1989).

Normal modes of vibration

A basic model to describe the interaction of the infrared radiation with matter is by looking to a molecule as a system of masses joined by bonds behaving like springs (figure 7). Taking the example of the diatomic molecules, these exhibit three degrees of translational freedom and two degrees of rotational freedom. The atoms in the molecules can also move relatively to one another, meaning that the bond lengths can vary, or one atom can move out of its present plane. Briefly, this is the definition of the bending and vibrational movements, and are referred collectively to as vibrations (Campbell et al., 2000, Bower and Maddams., 1989, Bower, 2002).

Figure 7 – Schematic representation of vibration modes in a diatomic molecule.

For a molecule consisting of N atoms, the number of vibrational degrees of freedom is $3N - 6$, leading to the same number of normal modes of vibration. For linear molecules, where low rotational modes are equivalent, the number of vibrational degrees of freedom is represented by $3N - 5$ (Campbell et al., 2000, Stuart, 2005). The degrees of freedom for polyatomic molecules, e.g. polymers, are summarized in table 2. Whereas a diatomic molecule has only one mode of vibration which corresponds to the stretching motion, a non-linear B-A-B type triatomic molecule has three modes, two of which correspond to stretching motions, with the remain corresponding to a bending motion. A linear type triatomic molecule has four vibration modes, two of which have the same frequency, and are said to be *degenerate* (Stuart, 2005).

Table 2 - Degrees of freedom for polyatomic molecules. (from (Stuart, 2005)).

<i>Type of degrees of freedom</i>	<i>Linear</i>	<i>Non-linear</i>
<i>Translational</i>	3	3
<i>Rotational</i>	2	3
<i>Vibrational</i>	$3N - 5$	$3N - 6$
<i>Total</i>	$3N$	$3N$

For a simple diatomic molecule, the vibration can be modelled as two masses, m_1 and m_2 , joined by a spring, with deformation obeying to the Hooke's law (equation 2). The vibration frequency is then expressed by:

$$\nu = \frac{1}{2\pi} \left(\frac{k}{\mu} \right)^{1/2} \quad (10)$$

where, k is the spring constant and μ is the reduced mass, given by:

$$\mu = \frac{m_1 m_2}{m_1 + m_2} \quad (11)$$

From equation 10 and 11, one can observe that a molecule can only absorb radiation when the infrared radiation reaching the sample is of the same frequency as one of the

fundamental modes of vibration of the molecule. In other words, a small part of the molecule has vibrational motion, while the rest is left unaffected.

Molecular vibrations can involve either changes in bond length - stretching, or bond angle - bending. Some bonds can stretch in-phase (symmetric stretching) or out-of-phase (asymmetric stretching) while the other vibrations contributing to the infrared are generically called as bending vibrations. Figure 8 presents the molecular vibrations that can occur in an infrared measurement of a polymeric material.

Asymmetric vibrations produce a displacement of the dipoles leading to a change in the dipole moment of the molecule, making these ones “infrared active”. On the other hand, symmetrical vibrations are weaker because they don’t produce a change in the dipole moment, and consequently usually are inactive in infrared (Campbell et al., 2000, Stuart, 2005).

Figure 8 – Representation of the most common infrared vibrations observed in a polymeric molecule.

A polymeric molecule can contain tens of thousands of atoms and consequently, the number of normal modes of vibration may be significantly higher. The infrared spectrum of a polymer may thus be expected to be tremendously complicated. However, polymeric materials are made from a large number of chemical identical units and many of the vibrations are equivalent, reducing the number of distinguishable vibrational states which simplifies the problem of assignments (figure 9) (Stuart, 2005, Bower and Maddams., 1989).

Figure 9 – Infrared spectrum of a fish gelatine sample.

The region between $1550 - 500 \text{ cm}^{-1}$ usually contains a very complicated series of absorptions, frequently overlapping each other, mainly due to all kinds of bending vibrations (figure 8). This area of the spectra is called the *fingerprint* region (figure 9) and is characteristic of each polymeric material, and could therefore be used to identify the chemical compound.

Infrared spectroscopy is a very useful and powerful characterization technique providing information about the chemical and physical structure of a polymer, because any two regions of the material that differ in the way the repeating units are arranged, may exhibit detectable differences in the IR spectra (Bower and Maddams., 1989,

Bower, 2002). In table 3 the most common vibrational modes found for polymers are summarized.

Table 3 - Characteristic vibration modes for common groups found in polymers. (Adapted from (Schrader, 2008, Campbell et al., 2000))

<i>Type of vibration</i>	<i>Characteristic frequency (cm⁻¹)</i>
O – H { <i>stretching</i> }	3650 – 3000
N – H { <i>stretching</i> }	3500 – 3300
C – H { <i>stretching</i> }	3200 – 2800
S – H { <i>stretching</i> }	2600 – 2550
C ≡ N { <i>stretching</i> }	2255 – 2220
C ≡ C { <i>stretching</i> }	2250 – 2100
C = O { <i>stretching</i> }	1820 – 1680
C = C { <i>stretching</i> }	1900 – 1500
C = N { <i>stretching</i> }	1680 – 1610
N = N { <i>stretching</i> } – aliphatic	1580 – 1550
N = N { <i>stretching</i> } – aromatic	1440 – 1410
C – NO ₂ { <i>stretching</i> }	1590 – 1530 {asymmetric} 1380 – 1340 {symmetric}
C – SO ₂ – C { <i>stretching</i> }	1350 – 1310 {asymmetric} 1160 – 1120 {symmetric}
C = S { <i>stretching</i> }	1250 – 1000
CH ₂ { <i>bending</i> }	1470 – 1400
CH ₃ { <i>bending</i> }	1380
C – C { <i>stretching</i> } – aromatic	1600, 1580, 1500, 1450, 1000
C – C { <i>stretching</i> } – alicyclic	1300 – 600
C – O – C { <i>stretching</i> }	1150 – 1060 {asymmetric} 970 – 800 {symmetric}
Si – O – Si { <i>stretching</i> }	1100 – 1000 {asymmetric} 550 – 540 {symmetric}
O – O { <i>stretching</i> }	900 – 845
S – S { <i>stretching</i> }	550 – 430

C – Cl { <i>stretching</i> }	800 – 550
C – Br { <i>stretching</i> }	700 – 550
C – I { <i>stretching</i> }	660 - 480

Quantitative analysis

When the infrared radiation passes through an absorbing medium (figure 10), it may be expressed in terms of the Lambert-Beer law:

$$A = \log \left(\frac{I_0}{I} \right) = kct \quad (12)$$

where A is the absorbance; I_0 is the incident light; I is the intensity at a thickness t in the absorbing medium; c is the concentration of absorbing species; and k is a constant for the material termed the absorptivity or extinction coefficient (Campbell et al., 2000).

Figure 10 – Illustration of Lambert-Beer law.

Values of absorbance range from zero, when there is no absorption, ($I = I_0$) to infinity when all incident light is completely absorbed ($I = 0$) (Campbell et al., 2000).

The IR absorption spectrum of a polymer sample is usually plotted as a percentage of the transmittance (I/I_0) or absorbance against a wavelength or wavenumber of infrared radiation. If the sample is very thick, the transmittance will approach zero and the absorbance will tend to infinity for all wavelengths. In order to avoid this issue, it is necessary to work with samples with thickness in the range of 30 – 300 μm (Bower, 2002).

Modern spectrometers are computer controlled, allowing not only to measure the IR spectra of the sample, but also to perform complex mathematical calculations of the data. As an example, they permit comparing the unknown polymer substance spectra with standard data of different polymers and other chemical substances present in the software database, ultimately leading to an identification of the polymer and its composition

Polymer molecular orientation

One of the most important features of infrared spectroscopy is the simplicity of the measurement, which is usually a non-destructive characterization technique, but also allows quantifying different material characteristics. The most important of those is the determination of polymer chain orientation, which will deeply influence the material performance.

As mentioned above, an infrared absorption band is associated with a particular vibration mode of the polymer bonds. For each one of these modes there is a particular direction within the polymer chain, which is the direction of the absorption dipole μ , also called *infrared-transition dipole* or *transition moment axis* (Bower and Maddams., 1989, Bower, 2002).

The IR radiation is absorbed by a particular functional group of the polymer chain only if the radiation frequency matches the frequency of vibration of the molecule, and a component of the electric vector E of the incident radiation I must be parallel to the transition-dipole axis. If a polymer chain is oriented, so the dipole axes should be oriented to, in other words, sample absorption will depend on the polarization of the incident radiation.

In order to determine the polymer chain orientation, infrared measurements of transmittance or absorbance must be performed with polarized IR radiation, parallel or perpendicular to the mechanical draw direction, for a particular absorption band of the spectrum. Figure 11 shows the FTIR spectra obtained for two orthogonal polarization directions, parallel and perpendicular to the draw direction of a poly(vinylidene fluoride) (PVDF) polymer sample. It is possible to observe that the overall spectra are similar with neither modes being totally suppressed nor new modes seeming to appear. Nevertheless, the amount of IR radiation absorbed by some particular modes are clearly different for IR spectra obtained with radiation polarized in the perpendicular or parallel to the polymer draw direction (figure 11).

Figure 11 – Detail of the FTIR spectra obtained for a PVDF uniaxial stretched sample. FTIR was collected with the IR radiation polarized in the polymer draw ($\parallel$) and perpendicular direction ($\perp$).

Assuming that reflectance and/or scattering do not contribute significantly to the differences in the attenuation and is only due to absorbance, the Lambert-Beer law

(equation 12) can be modified in order to obtain the absorbance for the two IR polarized directions:

$$A_{||} = k_{||}ct \quad (13)$$

$$A_{\perp} = k_{\perp}ct$$

where, $A_{||}$ and $A_{\perp}$ are the absorbance of the polarized light in the parallel and perpendicular to the polymer stretching direction, respectively: $k_{||}$ and $k_{\perp}$ are proportional to the average fractions of the incident energy per unit of area absorbed by a single absorber, for radiation polarized parallel or perpendicular to the polymer draw direction, respectively.

The absorption of the IR radiation is associated to changes in the oscillating dipole μ associated to the vibration mode. Considering that $A_{||} \propto \langle \mu_{||}^2 \rangle$ and $A_{\perp} \propto \langle \mu_{\perp}^2 \rangle$, where $\mu_{||}$ and $\mu_{\perp}$ are the components of the oscillating dipole in the parallel and perpendicular to the draw direction in the plane of the sample (figure 12).

Figure 12 – Definition of the angle between the transition dipole μ with respect to the long axis of the polymer molecule (Gedde, 2001) pag207.

Thus:

$$A_{||} = C \langle \cos^2 \theta_{\mu} \rangle \quad (14)$$

and assuming uniaxial symmetry:

$$A_{\perp} = C \langle \sin^2 \theta_{\mu} \cos^2 \theta_{\mu} \rangle = \frac{1}{2} C \langle \sin^2 \theta_{\mu} \rangle \quad (15)$$

where:

$$C = A_{||} + 2A_{\perp} = 3A_{iso} \quad (16)$$

where A_{iso} is the absorbance of an isotropic sample with same thickness and the simplification of equation 15 is due to the random orientation of the oscillating dipole μ (Bower, 2002). Replacing C in equation 14:

$$\langle \cos^2 \theta_\mu \rangle = \frac{A_{||}}{A_{||} + 2A_{\perp}} \quad (17)$$

The ratio between the $A_{||}$ and $A_{\perp}$ is called dichroic ratio, D :

$$D = \frac{A_{||}}{A_{\perp}} \quad (18)$$

Replacing D in equation 17, one obtains:

$$\langle \cos^2 \theta_\mu \rangle = \frac{D}{D + 2} \quad (19)$$

Thus, the polymer chains main orientation can be defined by the Herman's orientation function, f , given by:

$$f = \frac{(D - 1)(D_0 + 2)}{(D + 1)(D_0 - 2)} \quad (20)$$

where D_0 is the dichroic ratio for a sample with perfect uniaxial orientation and is dependent of the angle between the oscillating dipole μ of the considered vibrational band and the local chain axis segment of the polymer:

$$D_0 = 2\cot^2 \theta_\mu \quad (21)$$

Absorption bands associated with a perpendicular transition dipole moment have $D_0 = 0$, and the Herman's orientation factor is given by:

$$f = 2 \frac{(1 - D)}{(D + 2)} \quad (22)$$

One of the main attractions of the IR technique is that the dichroism of the absorption bands assigned to different groups, and orientation of the different groups of the monomer can be determined (Gedde, 2001). Herman's mathematical model predicts that related to the direction of interest, well aligned polymer chains had a value of 1, and perpendicular aligned chains had a value of -0.5, while for random aligned polymer chains $f = 0$, as illustrated in figure 13.

Figure 13 – Schematic representation of the Herman's orientation factor, related to polymer chain stretching direction.

The orientation of the uniaxial drawn PVDF was measured by Sencadas *et al.* (Sencadas et al., 2009). In their work, the vibrational modes at 765 and 510 cm^{-1} were chosen, because they are related to the crystalline α and β -phases of the polymer and both are very well defined CF_2 bond vibrations relative to the direction of the polymer chain, and the reorientation of the chains could be monitored independently, for each crystalline phase. In their work, they reported that there was a change of the Herman's orientation factor from 0, for a chain obtained from cooling the polymer from the melt, to -0.47 for the sample stretched five times its original length. By determining the position of the CF_2 dipole, the alignment of polymer chains in relation to the mechanical draw direction was indirectly measured. Their results were validated by measuring the polymer chain orientation by x-ray diffraction techniques (Branciforti et al., 2007), and the values reported are consistent with the ones obtained by FTIR measurements.

Thermal characterization techniques

Differential Scanning calorimetry

Differential scanning calorimetry (DSC) is a direct analytical experimental technique that measures the heat flux $\partial q / \partial t$ to or from a sample specimen as well as enthalpy

changes as a function of temperature or time. This method is not a passive experimental technique because there is a change of material structure, e.g. crystallinity, morphology, etc., during the measurement. In DSC, the samples can also be submitted to annealing, aging, curing, or even to erase the previous material thermal history (Kämpf, 1986).

The present discussion will focus on the physical principles of the measurement and how to interpret the recorded data under non-isothermal conditions. This technique is also useful to study the polymer crystallization kinetics; however, this topic is beyond the scope of this discussion. Fully detailed description and physical principles involved in polymer crystallization kinetics can be found in (Bower, 2002, Sperling, 2006).

The apparatus basically consists of two furnaces with controlled atmosphere and independent temperature controller, heating elements and individual thermocouples (figure 14). The sample and the reference sample (usually air) are placed inside small independent crucibles made from a high thermal conductive material, generally aluminium, but other materials can also be found such as gold, platinum, graphite or even copper.

Figure 14 – A schematic drawing of a differential scanning calorimeter. The number 1 represents the heating/cooling system and the number 2 exemplifies the thermocouples (Lobo and Bonilla, 2003).

The user-defined thermal program is applied to the sample and reference in the same conditions, especially the same heating and/or cooling rate, and the temperature of each pan is recorded independently. As an example, consider if the sample was ice, and a constant heating rate is applied for the reference and the sample. When the ice starts to melt, and during the entire melting process, the sample temperature remains constant while the reference temperature continues to increase. When the ice melting process finishes, the temperature of the sample starts to increase. By subtracting the temperature profile recorded for the sample and for the reference material, it is possible to calculate the amount of energy supplied to the sample during melting (figure 15).

Figure 15 – Diagram of the temperature profile applied to the reference and to the sample during a DSC measurement. T_r – temperature of the reference sample; T_s – temperature of sample (NETZSCH, 2015).

Despite being a destructive characterization technique, DSC is fast and easy to operate, only a small amount of sample (in the milligram scale) and a good physical contact between the sample and the base of the crucible is needed to reduce operation errors

(figure 16). The main sources of operation mistakes/errors during a DSC measurement are related to a bad calibration of the thermocouples, sample contamination from the surroundings, the presence of moisture and/or residual solvents and inaccurate sample mass.

Figure 16 – Sample preparation for a DSC experiment (NETZSCH, 2015).

Thermogram analysis

If a polymer sample is submitted to a heat cycle at constant heating rate and pressure, then the specific heat (C) of the polymer can be defined as:

$$C = \frac{Q}{m\Delta T} \quad (23)$$

where Q is the amount of heat added (heat flow), m is the mass of the sample and ΔT is the change in temperature. The heat of the melting (H_m) is defined as the amount of heat per unit of mass needed to change a substance from the solid state to a liquid state at its melting point. As the melting process is an endothermic reaction, it is represented by a positive peak in the DSC curve, considering the y-axis range setting of the heating flow (figure 17). The heat of melting can be calculated from the DSC plot by determination of the area under the melting transition, according to equation 24:

$$H_m = \frac{Q}{m} \quad (24)$$

From the analysis of the DSC plot (figure 17), one can acquire information from the different thermal events occurring in the polymer during the heating or cooling stages in the DSC measurement.

Figure 17 – Illustration of a DSC thermogram during the heating scan for a polymer sample. T_g - glass transition; T_c – cold-crystallization temperature; T_m - melting temperature.

In figure 17, the process that occurs at lower temperatures is called the glass transition (T_g). Below this temperature the polymer chains are in a “frozen” state and only local cooperative movements are allowed. When the temperature reaches the polymer glass transition, the movement of the polymer chains is promoted, softening the material, and is graphically represented by a slope/ shift in the baseline (represented by T_g in figure 17). The calculation of the glass transition temperature can be done in different ways. The most common is the half C_p or half-height of the shifted baseline as illustrated in figure 17 (Lobo and Bonilla, 2003).

After glass transition, increasing the temperature may lead to a cold-crystallization process (T_c) which is a thermal event observed for some polymers. This occurs due to the high mobility of the polymer chains that organize in a more packed structure, releasing energy of the system. It is an exothermic process and therefore is represented by a negative peak in the thermogram with the area of the cold-crystallization peak being proportional to the energy required to pack the polymer in a more organized state - ΔH_c (figure 17).

Finally, as temperature increases even further, the polymer crystalline regions reach the melting transition with the area of the melting peak being proportional to the amount of energy required to melt the crystalline regions of the polymer, also known as the melting enthalpy - ΔH_m , which can be used to calculate the percent of crystallinity present in the material.

The degree of crystallinity (X_c) for a given polymer is determined by applying the following equation:

$$X_c = \frac{\Delta H_m - \Delta H_c}{\Delta H_m^0} * 100 \quad (25)$$

where ΔH_m^0 is a reference value and represents the heat of melting for a 100% crystalline polymer sample. It has been established for most common engineering polymers and some of these are in table 2.4.

Depending on the sample thermal history, a cold-crystallization exothermic peak may or may not be observed during the DSC, and if it does not appear the value of $\Delta H_c = 0$.

Table 4 - heating of melting for a 100% crystalline polymer sample (adapted from (Mark, 2007))

Polymer	Acronym	ΔH_m^0 (J/g)
<i>Polypropylene</i>	PP	207.1
<i>Polyethylene</i>	PE	293.6
<i>Poly(ethylene terephthalate)</i>	PET	140.1
<i>Polyamide-6</i>	PA6	230.1
<i>Polyamide 12</i>	PA12	209.3
<i>Poly(lactic acid)</i>	PLA	93.0
<i>Poly(hydroxybutyrate)</i>	PHB	146.0

Thermogravimetric analysis

By definition, thermogravimetric analysis (TGA) is the measurement of the weight of a polymer sample as a function of temperature, time and/or atmosphere at a controlled temperature program. Usually, the evolution of the mass is measured in a high sensitive balance, under a well-defined atmosphere (inert or reactive gas) and plotted against temperature. Thermogravimetric analysis is used not only for polymeric materials, but also to characterize materials used in various environmental, food, pharmaceutical, and petrochemical applications. An example of a TGA plot is presented in figure 18.

The thermogram in figure 18 reveals several information parameters related to polymer degradation. In the case of poly(lactic acid) (PLA), the mass remains constant until pyrolysis starts to occur. The initial degradation temperature ($T_{initial}$) is defined as the temperature at which the material degradation begins, and is represented in the thermogram as the point at which there is a sudden decrease in mass from the initial sample weight. This temperature is particularly important because it gives information about the highest temperature that a polymer can be processed. Nevertheless, one should take in account that initial mass losses can be observed due to the presence of moisture in the sample (Machado et al., 2015). The onset temperature (T_{onset}) is calculated by extending the pre-degradation portion of the curve to the point of the interception with a line drawn as a tangent to the steepest portion of the mass curve occurring during degradation (Turi, 2012). As represented in figure 18 it refers to the temperature where oxidation just begins. Some features in the TGA curve may not be readily perceived and

can be more easily detected by applying a first-order derivative in the thermogram. The derivative thermogravimetric (DTG) curve is the best indicator of the temperature at which the various stages of thermal decomposition take place and provides information about the decomposition rates. The maximum of the reaction rate ($T_p = \partial m / \partial T$) is obtained by the peak of the DTG and indicates the point of greatest rate of change on the weight loss curve (Lobo and Bonilla, 2003, Turi, 2012).

The residual mass ($M_{residual}$) obtained in the end of the thermogravimetric experiment is usually attributed to ashes resulting from the sample pyrolysis process.

Figure 18 – TGA thermogram and derivative obtained for a poly(lactic acid) at a heating rate of 20°C/min under nitrogen atmosphere.

Thermal degradation kinetics

From the TGA data, the kinetics of the thermal degradation process can be studied. A typical model for the thermal decomposition rate ($-\frac{\partial(1-\alpha)}{\partial t}$) of a homogeneous system follows the general expression:

$$-\frac{\partial(1-\alpha)}{\partial t} = k(T)f(\alpha) \quad (26)$$

where, $f(\alpha)$ represents the net result of elementary steps, as the polymer thermal degradation is often a chain reaction, $k(T)$ is the rate constant and α is the of conversion of the sample under degradation, and is defined by:

$$\alpha = \frac{w_0 - w(t)}{w_0 - w_\infty} \quad (27)$$

where w_0 , w_∞ and $w(t)$ are the weight of the sample before degradation, at a given time (t), and after complete degradation, respectively. The rate constant has an Arrhenius behavior with the absolute temperature:

$$k = Ae^{\left(\frac{-E_{act}}{RT}\right)} \quad (28)$$

where A is the pre-exponential factor, E_{act} is the activation energy of the degradation process, R is the gas constant and T is the absolute temperature.

Substituting equation 28 in equation 26, one obtain:

$$-\frac{\partial(1-\alpha)}{\partial t} = Ae^{\left(\frac{-E_{act}}{RT}\right)} f(\alpha) \quad (29)$$

If the temperature of the sample is changed by a constant value of β , being $\beta = \partial T / \partial t$, the variation of the degree of decomposition can be analyzed as a function of temperature. Therefore, the decomposition rate is given by:

$$-\frac{\partial(1-\alpha)}{\partial t} = \frac{A}{\beta} e^{\left(\frac{-E_{act}}{RT}\right)} f(\alpha) \quad (30)$$

Based in these mathematical expressions, several empirical models were developed to characterize and study the degradation kinetics for polymeric materials, being the most used the Ozawa-Flynn-Wall and Kissinger methods.

(a) *Ozawa-Flynn-Wall (OFW) model*: this isoconversional method is an integral method. Rearranging and integrating equation 30, it can be obtained:

$$g(\alpha) = \int_{\alpha_0}^{\alpha_p} \frac{\partial \alpha}{f(\alpha)} = \frac{A}{\beta} \int_{\alpha_0}^{\alpha_p} e^{\left(\frac{-E_{act}}{RT}\right)} \partial T \quad (31)$$

where α_p is the maximum conversion. Defining $x = \frac{E_{act}}{RT}$ (Ou et al., 2010) and integrating the right-hand side of the equation 31:

$$\frac{A}{\beta} \int_{\alpha_0}^{\alpha_p} e^{\left(\frac{-E_{act}}{RT}\right)} \partial T = \frac{AE_{act}}{\beta R} p(x) \quad (32)$$

After taking the logarithms, we get:

$$\log(\beta) = \log \frac{AE_{act}}{g(\alpha)R} + \log(p(x)) \quad (33)$$

Doyle demonstrated that the $\log(p(x))$ for polymer systems, obeys to the following expression (Ou et al., 2010, de Britto and Campana-Filho, 2007, Doyle, 1962):

$$\log(p(x)) = -2.315 - 0.4567x \quad (34)$$

Replacing in equation 33, the general expression for the OFW can be obtained:

$$\log(\beta) = \log \frac{AE_{act}}{g(\alpha)R} - 2.315 - \frac{0.4567E_{act}}{RT} \quad (35)$$

The values of the activation energy over a wide range of decomposition can be obtained by plotting $\log(\beta)$ vs $1/T$, at a constant value of the conversion rate. The slope of each line is $-\frac{0.4567E_{act}}{RT}$.

(b) *Kissinger model*: this method relies on experiments performed at different heating rates, and is expressed by:

$$\ln\left(\frac{\beta}{T_p^2}\right) = \left\{ \ln \frac{AR}{E_{act}} + \ln \left[n(1 - \alpha_p)^{n-1} \right] \right\} - \frac{E_{act}}{RT_p} \quad (36)$$

Where T_p is the temperature of the maximum reaction rate, and generally is obtained by the minimum of the derivative, according to figure 18. The plot of $\ln\left(\frac{\beta}{T_p^2}\right)$ vs $\frac{1}{T_p}$ will yield the straight line whose slope is $\frac{E_{act}}{R}$.

Both methods here described allows the calculation of the thermal degradation activation energy of a polymer system without the knowledge of the thermal degradation reaction order.

Scanning electron microscopy

Scanning electron microscopy (SEM) is a very powerful and versatile technique used in materials science with a major impact on the applications of microscopy due to the variety of surfaces that can be observed using a relatively simple approach.

The SEM equipment uses a beam of high energy electrons generated by an electron gun that is processed by magnetic lenses, focused at the surface of the specimen and rastered across the sample surface (Facility, 2016). In this technique, the image is a result of the beam probe illuminating the sample one point at a time in a rectangular scanning pattern, with the strength of the signal generated from each point being a reflection of differences (e.g. topographical or compositional) in the sample. There is a one-to-one relationship between the number of points on the specimen and points on collected image screen. In general, an increase of the magnification leads to a decrease of the size of the scanned area. Figure 19 presents a scheme of the general SEM layout and its function.

Figure 19 - Scanning electron microscope layout and function (Facility, 2016).

The interaction between the electrons and the sample produces various signals, including secondary electrons, backscattered electrons, characteristic X-rays, light (cathodoluminescence), specimen current and transmitted electrons (figure 20). They provide information about sample morphology, topography, chemical composition, among other material information (Facility, 2016).

Backscattered electrons are promoted by elastic interaction with sample atoms, and are send back almost in the same direction as the one they came from, and with a very little energy loss. Chemical elements with higher atomic number produce more backscattered electrons than the ones that have a low atomic number, which enable to differentiate the chemical elements or phases present in a sample, according to their atomic number. Generally, atoms with a higher atomic number appears as white and with lower atomic number looks as black (Kuo, 2007).

Secondary electrons result from the inelastic interaction between the high energy electrons produced by the beam with the valence electrons of atoms in the sample, which cause the ejection of the electrons from the atoms. Secondary electrons have lower energy, less than 50 eV, and are independent of the atomic number of the

scattering electrons (unlike the situation for backscattered electrons). Images obtained with the detection of secondary electrons mostly represent the sample topography (Amelinckx et al., 2008, Smith, 1990).

Figure 20 – Signals that are coming from the sample from the interaction of an electron beam with a bulk sample.

The microscope electron beam is able to remove electrons from the various electronic layers of the atoms present in the observed sample. When an electron is ejected, it is replaced by other from an upper layer, and a photon with energy equal to the energy difference between the two layers is emitted, and the process will continue until the last layers of electrons have been replaced, and all characteristic rays of the atom will be emitted. The X-ray detector present in the SEM equipment is able to determine the quantity of photons energy that hits the detector, and a histogram can be drawn with the photons energy versus the number of photons received, and thanks to the database, the spectrum interpretation is facilitated, thereby making possible to obtain the list of elements present in the sample (Kuo, 2007, Amelinckx et al., 2008).

Sample Preparation for SEM Analysis

The SEM experiment will only work if the sample is prepared in a proper manner. While metals do not require any particular preparation, as they already conduct electricity when they are bombarded with electrons, and current flows throughout the sample. However, ceramic and polymeric materials, especially biological samples, need an extra thin layer of a conductive material on the surface of the sample, in order to avoid electron charge that can lead to a local increase of temperature, interacting with the sample, and consequently to the loss of the original specimen morphology (Kuo, 2007).

The thin layer of conductive material, usually gold, gold-palladium alloy or even amorphous carbon, is generally deposited by sputter coaters. Biological samples can absorb moisture from the environment and they need to be carefully stored in order to avoid adsorbed water molecules in the material. If present, these molecules will

vaporize in a vacuum chamber, creating obstacles for the electron beams and obscuring the clarity of the image (Amelinckx et al., 2008, Hunter et al., 1993).

New SEM equipment's no longer require a full vacuum to operate, nevertheless, the image quality produced have weaker resolution, but they open up the possibility to observe the sample without an extra conductive layer external to the material, and consequently obtain a morphology close to the real sample (Kuo, 2007, Facility, 2016).

The Applications of Scanning Electron Microscopy

Since their invention in 1935, this powerful technique opened the possibility to observe an entire new World in scientific fields from physics, to engineering and biology, allowing scientists to access new, and useful information about material microscopic properties and correlate to materials macroscopic features.

The main application of SEM equipment is to observe sample morphology, porosity, crystal and grain size orientation and some bonding differences, through contrast and using backscattered electrons (figure 20). Nevertheless, combined with Energy-Dispersive X-Ray Spectroscopy (EDS) it is a useful technique for the production of material elemental maps, representing accurately the distribution of chemical elements present within the samples. With this equipment, it is possible to remove material from samples, cutting pieces out or remove progressive slices from samples, using a focused ion beam.

With a cryo-scanning electron microscope, it is possible to freeze-dry a sample inside of the equipment, coating with a thin layer of conductive material, and observe the morphology that material has when is immersed in a liquid.

Bioinspired materials are often processed in the shape of micro and nanofibers by a number of techniques such drawing, template synthesis, phase separation, self-assembly and electrospinning. This fibrous shape, have the ability to tailor fibre mats which resembles native extracellular matrix (ECM), concerning its nanostructure, biochemical cues and morphology. For instance, this feature is of outmost importance since the alignment of the substrate nanofibers seems to be directly related with oriented cell growth (Sundararaghavan et al., 2013).

Fibre alignment within the mats can be quantified through the analysis of sample morphology acquired by SEM. A method based in the analysis of the images by Fast Fourier Transform (FFT), which converts the information present in the original SEM

image from the “real” space into mathematically defined “frequency” space was developed. The resulting FFT output image contains grayscale pixels that are distributed in a pattern that reflects the degree of fibre alignment present in the selected area (Alexander et al., 2006, Ayres et al., 2006). This procedure was developed for open source software Image J (Schneider et al., 2012) and allows to quantify the overall alignment of fibres, and the angular distribution (Valente et al., 2016), and utterly correlate with cell morphology when placed in the presence of such material morphologies.

Technological development gives us today a fast, reliable and an easy equipment to operate and to obtain accurate material visual information, being one of the most useful characterization techniques available.

An SEM virtual laboratory were one can simulate the equipment operation, acquiring expertise, and many other useful information is available on Australian Microscopy and Microanalysis Research facility (<http://www.ammrf.org.au/myscope/sem/introduction>) (Facility, 2016).

Cytotoxicity testing

A biomaterial is defined as a material that exists in contact with tissues of the human body without causing an unacceptable degree of harm to that body (Williams, 2008). In the development of biomaterials there are several parameters that need to be addressed before considering its potential application. Factors like biocompatibility, cell attachment and proliferation and biodegradation are of great importance as they relate to the biological performance of a given material.

According to Williams (Williams, 2008), biocompatibility is defined as “the ability of a biomaterial to perform its desired function with respect to a medical therapy, without eliciting any undesirable local or systemic effects in the recipient or beneficiary of that therapy, but generating the most appropriate beneficial cellular or tissue response in that specific situation, and optimizing the clinically relevant performance of that therapy”. A broader definition is proposed by Vert *et al.* (2012) following the IUPAC (International Union of Pure and Applied Chemistry) recommendations in which, the biocompatibility of a given polymer is defined as the “ability to be in contact with a living system without producing an adverse effect”. Nevertheless, one can easily figure out that

evaluation of biocompatibility is a complex task generally involving animal experiments and long term evaluation after implantation.

Among the biocompatibility tests, cytotoxicity is preferred and is routinely evaluated using *in vitro* methodologies as a preliminary screening for biocompatibility assessment. Despite presenting several disadvantages such as neglecting the diversity of medical devices, the variability of the environment of the body and the complexity of the interaction between the body and the biomaterial (Li et al., 2015), cytotoxicity tests are one of the most important methods for biological evaluation.

Cytotoxicity testing is simple, fast and highly sensitive, representing an alternative to the use of animals while providing valuable initial indications to determine whether a biomaterial exhibit either cytotoxicity or cytocompatibility with living cells. Evaluation of cytotoxicity usually involves chemical substances that can be delivered to a cell culture in a defined dose. Consequently, the assessment of a biomaterial's cytotoxicity represents a challenge because biomaterials (and the resulting biomedical devices) are made of solid materials, creating extra difficulties or turning impossible to measure the specific dose of a substance of interest.

Biomaterials/devices are generally composed from relatively inert, high molecular weight materials and it is their leachable components (e.g. contaminants, additives, fillers, residual compounds derived from manufacturing) that usually present cytotoxicity. As a result, the rationale behind the *in vitro* testing of biomaterials is that cytotoxicity can be assessed by evaluating the impact of constituents that can be released from the biomaterial/device in *in vivo* conditions.

The Standard ISO 10993 describes a series of procedures for evaluating the biocompatibility of medical devices therefore, for testing solid samples. There are three main categories of tests stated in ISO 10993-5 (International Organization for Standardization, 2009) which relates to general tests for *in vitro* cytotoxicity: i) test on extracts, ii) test by direct contact and iii) test by indirect contact. These methods are designed to determine the biological response of mammalian cells following incubation of cultured cells in contact with extracts of the test sample or with a device/biomaterial. The following sections summarize essential ISO procedures for cytotoxicity testing (International Organization for Standardization, 2009, International Organization for Standardization, 2004).

Reference materials

Experimental controls namely, negative controls, blanks and positive controls should be used in all biological evaluations to accurately validate a certain procedure and compare the results between materials. Some examples of materials that may be used as positive and negative controls for cytotoxicity assays are listed in Table 5.

Table 5 – Materials that have been used as negative and positive controls for cytotoxicity assays according to ISO 10993-12 (International Organization for Standardization, 2004).

<i>Material</i>	<i>Negative Control</i>	<i>Positive Control</i>
<i>High-density polyethylene</i>	●	
<i>Low-density polyethylene</i>	●	
<i>Silica-free polydimethylsiloxane</i>	●	
<i>Polyvinylchloride</i>	●	
<i>Polyetherurethane</i>	●	
<i>Polypropylene</i>	●	
<i>Aluminium oxide ceramic rods</i>	●	
<i>Stainless steel</i>	●	
<i>Commercially pure titanium alloys</i>	●	
<i>Polyvinylchloride with organotin additives</i>		●
<i>Polyurethane films containing zinc diethyldithiocarbamate</i>		●
<i>Polyurethane films containing zinc dibutyldithiocarbamate</i>		●
<i>Certain latex formulations</i>		●
<i>Solutions of zinc salts</i>		●

<i>Copper</i>		•
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Preparation of extracts

For the preparation of liquid extracts of a material, the extracting conditions should simulate the conditions of the intended clinical use. For this purpose, the extracting solutions (e.g. culture medium with or without serum, water, physiological saline, freshly refined vegetable oil, ethanol/water, ethanol/saline, diluted polyethylene glycol 400, dimethyl sulfoxide) should be chosen by taking into account the final application, should be compatible with the cell culture and not affect the extraction temperature. Furthermore, the concentration of the extraction vehicle should be compatible with the culture system and not induce toxicity of the testing cells.

Having chosen the most adequate extracting vehicle, the extraction is often prepared at the highest temperature compatible with the physical characteristics of the extracting vehicle and sample (i.e. without causing physical changes in the material) in order to maximize the amount of extractable substances as well as to simulate the highest temperature the biomaterial may be exposed. Moreover, the applied temperature should not exceed the glass transition temperature of the biomaterial or cause hydrolysis, as these can affect the amount and type of extractables (International Organization for Standardization, 2004).

Extraction conditions based on common practices are presented in table 6. Nevertheless, other conditions that better simulate the end application and may occur during clinical use, such as prolonged or shortened extraction times, may be used. As for extracting conditions and although most of extractions are performed under static conditions, these should be performed in conditions close to the end use that is, agitation should be considered when appropriate. Importantly, according to the ISO 10993-12 recommendations (International Organization for Standardization, 2004), the samples prepared for cytotoxicity testing should be properly sterilized to avoid contamination of the cell culture.

Table 6 – Extraction conditions based on common practices (International Organization for Standardization, 2009).

<i>Extraction Time [h]</i>	<i>Extraction Temperature [°C]</i>
24 ± 2	37 ± 1

72 ± 2	50 ± 2
24 ± 2	70 ± 2
1 ± 0.2	121 ± 2

As noted above, extraction is a complex process influenced by parameters such as time, temperature and extraction medium.

Another factor that needs to be considered during the extraction process is the surface-area-to-volume ratio (International Organization for Standardization, 2004). Before extraction and according to ISO 10993-12 recommendations, the materials should be cut into small pieces such as 10 mm x 50 mm or 5 mm x 25 mm to enhance submersion in the extracting medium. Table 7 presents the standard surface area and the corresponding volume of extracting solution needed, however in case of impossibility to determine the surface area, a mass/volume of extracting solution should be used. Nevertheless, other surface-area-to-volume extraction ratios can be used if they simulate the clinical end use conditions.

Table 7 – Standard surface areas and extraction solution volumes (International Organization for Standardization, 2004).

<i>Thickness</i> <i>[mm]</i>	<i>Extraction ratio</i> <i>(surface area or mass/volume)</i> $\pm 10 \%$	<i>Forms of material</i>
< 0.5	6 cm ² /mL	film, sheet, tubing wall
0.5 to 1.0	3 cm ² /mL	tubing wall, slab, molded items
> 1.0	1.25 cm ² /mL	larger molded items
Irregularly shaped solid devices	0.2 g/mL	powder, pellets, foam, non- absorbent, moulded items
Irregularly shaped porous devices (low- density materials)	0.1 g/mL	membranes

Preparation of material for direct-contact assay

For the direct-contact tests, it is required the used of materials with at least one flat surface although the materials can have various shapes, sizes or physical states (e.g. liquid, solid, gel). An important factor to consider is the sterility of the sample that must be maintained throughout the test procedure as, for example, bacterial contamination can lead to false evaluation of cytotoxicity. In this regard, the effect of sterilization on the biomaterial/device should also be considered as it can alter the properties of the material. In the case of liquid samples, these can be tested either by direct deposition of the sample or by deposition in biologically inert absorbent matrices such as filter discs.

Assessment of cytotoxicity according to ISO standards

Cell cytotoxicity may be assessed either qualitatively or quantitatively by observing changes in cell morphology, as well as by assessing changes in viable cell populations. However, one should take in account that ISO 10993-5 recommends the use of quantitative assays over qualitative methods. In the qualitative evaluation, cell cytotoxicity can be determined microscopically with the optional use of cytochemical or vital stains by assessing changes in general morphology, vacuolization, cell detachment, cell lysis and membrane integrity (tables 8 and 9). Qualitative assessment of cytotoxicity may be measured or estimated using descriptive terms (e.g. none, slight, mild, etc.) or semi-quantitative numeric terms (e.g. 0, 1, 2, etc.). ISO 10993-5 recommends that a grade higher than 2, based on tables 8 and 9, is considered to come out from a cytotoxic effect.

Vital stains are useful to discriminate between live and dead or injured cells on the basis of membrane permeability whereas cytochemical stains are useful to identify specific cellular components (Rana, 2008). Examples of vital stains include neutral red which is taken by the lysosomes of healthy viable cells, and trypan blue which is taken up by dead cells as the dye only crosses the membrane of dead cells. The cytochemical staining depends on the chemical composition or the enzymatic activity of the stain component and therefore, cytochemical stains are used to assess the presence/absence of specific organic compounds and their localization (Rana, 2008).

The quantitative evaluation of cytotoxicity measures cell death, inhibition of cell growth and cell proliferation by assessing the number of cells, amount of protein, release of enzymes, release of vital dye, reduction of vital dye, etc. One should take into account

that according to the ISO 10993-5 recommendation, a reduction of cell viability of more than 30% may be attributed to a cytotoxic effect.

Table 8 – Morphological qualitative graded scale for test on extracts assay (International Organization for Standardization, 2009).

Grade	Reactivity	Conditions of all cultures
0	None	Discrete intracytoplasmatic granules, no cell lysis, no reduction of cell growth.
1	Slight	Not more than 20 % of the cells are round, loosely attached and without intracytoplasmatic granules, or show changes in morphology; occasional lysed cells are present; only slight growth inhibition observable.
2	Mild	Not more than 50 % of the cells are round, devoid of intracytoplasmatic granules, no extensive cell lysis; not more than 50 % growth inhibition observable.
3	Moderate	Not more than 70 % of the cell layers contain rounded cells or are lysed; cell layers not completely destroyed, but more than 50 % growth inhibition observable.
4	Severe	Nearly complete or complete destruction of the cell layers.

Table 9 – Graded scale for direct and indirect contact tests (International Organization for Standardization, 2009).

Grade	Reactivity	Description of reactivity zone
0	None	No detectable zone around or under specimen.
1	Slight	Some malformed or degenerated cells under specimen.
2	Mild	Zone limited to area under specimen.
3	Moderate	Zone extending specimen size up to 1.0 cm.
4	Severe	Zone extending farther than 1.0 cm beyond specimen.

1.5.1 Test on extracts

This test determines if a biomaterial extract (a solution containing the extractable compounds of a biomaterial) has potential to cause cell morphology changes and/or lysis and is generally suitable for detecting the toxicity of soluble substances of medical devices. This assay allows for both quantitative and qualitative assessment of cytotoxicity and is usually consistent with animal toxicity tests. In the standard assay (Figure 21), a cell suspension is evenly distributed over the surface of a tissue culture disk and incubated at 37 ± 1 °C with or without carbon dioxide depending of the culture conditions, and allowed to attach until reaching a near-confluent monolayer of cells. The culture medium is removed and replaced with fresh medium containing the extract sample, and a toxicity assay is performed usually for a period of 24 to 72 hours. The toxic substances present in the extract will be evenly distributed within the culture medium, resulting in a consistent pattern throughout the plated cells. Cytotoxicity is then evaluated for example, by measuring live and dead cells as previously described. Qualitative assessment of cytotoxicity based on morphological changes is recorded in a graded scale as the example provided in table 8.

Figure 21 - Schematic representation of test on extracts assay.

1.5.2 Test by direct contact

Similarly, to the tests on extracts, the direct contact assays allows for both qualitative and quantitative assessment of cytotoxicity. This test determines if a material itself has

potential to be cytotoxic and is the most sensitive, allowing the measurement of toxicity in biomaterials even at low levels. In this test, the cultured mammalian cells are in direct contact with the biomaterial (figure 22). This test is dependent on the diffusion of substances from the testing biomaterial into the culture medium and thus, extreme care should be put in sample handling to avoid disturbing the cell layer as this may induce a physical trauma to the cells and lead to a misinterpretation of cell death as cytotoxicity response. In the same way as in the extracts assay, a cell suspension is evenly distributed over the surface of a tissue culture disk and incubated at 37 ± 1 °C with or without carbon dioxide depending of the culture conditions, and allowed to attach until reaching a near-confluent monolayer of cells. The culture medium is removed and replaced with fresh medium and the testing material sample is carefully placed in the centre of the cell layer ensuring that at least one tenth of the cell culture is covered by the sample. The cell cultures are then incubated for 24-48 hours at the same conditions of the initial cell propagation, and cell viability is assessed after discarding the supernatant culture medium for example, through the use of vital stains. Qualitative assessment of cytotoxicity may be measured or estimated using descriptive terms (e.g. none, slight, mild, etc.) or semi-quantitative numeric terms (e.g. 0, 1, 2, etc.) as described in table 9.

Figure 22 - Schematic representation of direct contact assay.

1.5.3 Test by indirect contact

This test determines if a material itself has potential to release agents that may be cytotoxic and is suitable for biomaterials with large toxicity. The indirect contact test includes the agar overlay assay and filter diffusion, giving a qualitative assessment of cytotoxicity (table 9).

Agar diffusion Assay

In contrast to placing the testing material directly on a monolayer of cells as in the direct contact test, the agar diffusion assays use a monolayer of cells with a semi-solid agar or agarose overlay (3-4 mm) on which the material sample is placed (figure 23). Agarose

is generally preferred since the gelling point is between 30-34 °C whereas, the gelling point of agar is generally at 40-45 °C which contributes to a thermal shock during the overlay process. Like the direct contact test, this assay is based on the diffusion of substances into the culture medium. In the standard assay and as in the other cytotoxicity tests, a cell suspension is evenly distributed over the surface of a tissue culture disk and incubated at 37 ± 1 °C with or without carbon dioxide depending of the culture conditions, and allowed to attach until reaching a near-confluent monolayer of cells. The culture medium is removed and an appropriate volume of fresh medium containing serum with melted agar at a concentration of 0.5% to 2% is added. The agar/medium mixture should be in liquid state and at a temperature compatible with cells to avoid cell damage that may be inadvertently misinterpreted as a cytotoxicity response.

After solidification of the agar layer the testing material sample is placed in the centre ensuring that at least one tenth of the cell layer surface is covered by the sample. The cell cultures are then incubated for 24-72 hours at the same conditions of the initial cell propagation after which, the testing material is carefully removed from the agar layer. If desired, vital dyes may be incorporated into the agar or agarose for analysis before and after incubation with the testing biomaterial. However, if the vital dye is added before incubation, care should be employed to protect the culture from light as the photoactivation of the stain can lead to cell damage.

Cytotoxicity is qualitatively evaluated using a semi-quantitative graded scale (table 9). Importantly, as the agar diffusion test is a barrier model since agents must diffuse through the agar before contacting with pleated cells, this assay is not appropriate for leachables (definition below) that cannot diffuse through the agar layer, or that may react with agar.

Figure 23 - Schematic representation of the agar diffusion assay.

Filter diffusion Assay

The filter diffusion assay is another barrier model assay that gives a qualitative assessment of cytotoxicity. In the standard test, a surfactant-free filter with 0.45 µm of pore size is placed in a culture dish. A cell suspension is evenly distributed over the

surface of the filter and incubated at 37 ± 1 °C, with or without carbon dioxide depending of the culture conditions, and allowed to grow to near confluency at the end of the logarithmic phase of the growth curve. The medium is removed and the filters transferred with cell side down onto a layer of solidified agar as described for the agar diffusion assay (medium with agar at a concentration of 0.5% to 2%). The sample of the biomaterial to be tested is placed in the top of the filter, in the acellular side, with non-reactive rings delimitating the area in order to retain any liquids.

Following incubation at the same conditions of the initial cell propagation for $2 \text{ h} \pm 10$ min, the material samples are carefully removed from the filter and the filter separated from the agar surface. Cells are stained with adequate dyes and qualitatively evaluated using a semi-quantitative graded scale (table 9).

Further considerations

According to ISO 10993 and as described above, cytotoxicity assessment is performed using a solution containing the compounds (extractables) that are released from the biomaterial under aggressive conditions, such as elevated temperature, extended contact time or aggressive solvent system. Under normal conditions of exposure, the compounds that migrate from the surface of a material to a drug product formulation as a result of direct contact with the formulation, are defined as leachables. For example, considering a closed container system or a packaging material with a drug content, the extractables are the chemical species that migrate from the container into the contents when exposed to aggressive conditions and the leachables are the chemical species that make their way into the product under normal application conditions. Although leachables may be classified as a subset of the extractables, they can interact with the drug product originating new components. Therefore, in the evaluation of cytotoxicity of biomedical devices containing active products/drug formulations, it is advised to conduct an examination of both the extractable and leachable substances.

Sources of further information

This chapter is focused in the main general techniques that are useful to characterize and understand the mechanical, chemical and thermal properties of materials. Nevertheless, other characterization techniques can be used to assess a particular feature of the

material in study, in order to correlate the properties of the material to its structure, and processing technique. The following books are a good source for general reading:

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Figures:

Figure 24 – Relationship between the materials properties and the characterization.

Figure 25 – Representative stress-strain plot for a polymeric material. The numbers in parentheses represent the different stages that occur during mechanical experiment in polymeric samples.

Figure 26 – Schematic stress-strain curves of different polymers, drawn approximately to scale (Bower, 2002).

Figure 27 - Fatigue testing (Ashby and Jones, 2012)

Figure 28 – Schematic of a mechanical hysteresis loop.

Figure 29 – a) PCL sample microstructure with fibrin filling the pore structure, b) sample characteristic hysteresis loops with inset showing the cyclic mechanical loading applied, c) Relationship between the overall equivalent behaviour similar to plastic strain energy density and number of load-recovery cycles of PCL samples and d)

Comparison of predicted with experimentally fatigue behaviour calculated according to the Morrow's model (figure adapted from (Panadero et al., 2013)).

Figure 30 – Schematic representation of vibration modes in a diatomic molecule.

Figure 31 – Representation of the most common infrared vibrations observed in a polymeric molecule.

Figure 32 – Infrared spectrum of a fish gelatine sample.

Figure 33 – Illustration of Lambert-Beer law.

Figure 34 – Detail of the FTIR spectra obtained for a PVDF uniaxial stretched sample. FTIR was collected with the IR radiation polarized in the polymer draw ($\parallel$) and perpendicular direction ($\perp$).

Figure 35 – Definition of the angle between the transition dipole μ with respect to the long axis of the polymer molecule (Gedde, 2001) pag207.

Figure 36 – Schematic representation of the Herman's orientation factor, related to polymer chain stretching direction.

Figure 37 – A schematic drawing of a differential scanning calorimeter. The number 1 represents the heating/cooling system and the number 2 exemplifies the thermocouples (Lobo and Bonilla, 2003).

Figure 38 – Diagram of the temperature profile applied to the reference and to the sample during a DSC measurement. T_r – temperature of the reference sample; T_s – temperature of sample (NETZSCH, 2015).

Figure 39 – Sample preparation for a DSC experiment (NETZSCH, 2015).

Figure 40 – Illustration of a DSC thermogram during the heating scan for a polymer sample. T_g - glass transition; T_c - cold-crystallization temperature; T_m - melting temperature.

Figure 41 – TGA thermogram and derivative obtained for a poly(lactic acid) at a heating rate of 20°C/min under nitrogen atmosphere.

Figure 42 - Scanning electron microscope layout and function (Facility, 2016).

Figure 43 – Signals that are coming from the sample from the interaction of an electron beam with a bulk sample.

Figure 44 - Schematic representation of test on extracts assay.

Figure 45 - Schematic representation of direct contact assay.

Figure 46 - Schematic representation of the agar diffusion assay.